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Report Number I.

Regulation of staphylococcal enterotoxin biosynthesis

Annual Report

John J. Iandolo

February 1981

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Transformation	Immunoelectrophoresis	
Plasmid	Methicillin	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The genetics of enterotoxingenesis in S. aureus is being pursued. Enterotoxin B (SEB) has been shown to exist as both a chromosomal and plasmid gene in methicillin resistant strains. Most of these exhibit chromosomal genes although 37.5% possess a 1.15 Mdal plasmid which codes for SEB. Either genetic arrangement appears genetically linked to methicillin resistance although actual physical linkage has been ruled out. The association seems to be mediated by a tetracycline resistance plasmid.

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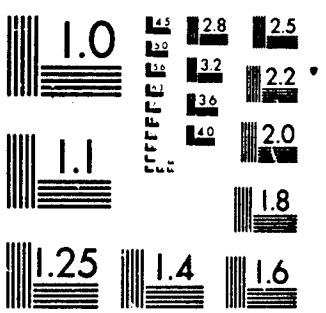


1. Summary:

The objectives of this research project are to genetically and biochemically extend our understanding of the regulation of the enterotoxins produced by the staphylococci. In particular, we need 1) to know why enterotoxin B (SEB) exists as a plasmid gene in some strains and a chromosomal gene is other strains 2) why either genetic conformation of SEB is intimately associated with both methicillin (mec) and tetracycline (tc) resistance for establishment and transfer 3) how tetracycline plasmids (and possibly other small plasmids) strongly influence the expression of the SEB gene, entB 4) to extend genetic scudies to include the 5 described serotypes of staphylococcal enterotoxin. The methods of investigation will rely on transformational and transductional analysis to obtain genetic data; on plasmid characterization by restriction mapping and cloning and by in vitro protein synthesis of enterotoxin genes.

A summary of results obtained thusfar is as follows:

1) Plasmid DNA analysis of 16 mec^r SEB^+ isolates showed that the entB plasmid was present in only 6 strains (37.5%). All of the other isolates contained typical penicillinase plasmids and smaller plasmids responsible for tetracycline or chloramphenicol resistance.

2) Genetic analysis of two strains (S. aureus DU-4916 and 592) which contain the 1.15×10^6 dalton entB plasmid confirmed that the toxin gene was extrachromosomal while similar studies of isolates which lack the entB plasmid demonstrated that the gene was chromosomal.

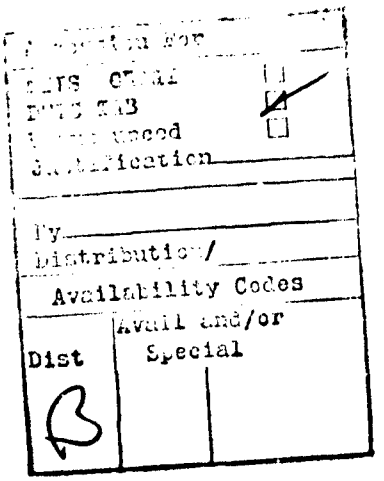
3) Transduction and transformation of mec^r to sensitive strains did not reveal linkage of mec and entB. However, both genes could be cotransduced at low frequencies with a plasmid responsible for resistance to tetracycline. We have proposed that the tetracycline resistance plasmid may act as an intermediate vector in the establisment of the mec^r SEB^+ phenotype. The data presented in the enclosed manuscript (appendix 1) suggests that although these 3 genetic elements are not linked they are capable of a transient interaction.

4) Transduction of entB into a recombination deficient host showed that only plasmid entB genes could be so transferred. Chromosomal entB was not established. This finding suggests that chromosomal entB does not possess the ability to translocate. However, because of the inability to directly select for entB further evaluation of transposability is needed. These data are presented in the manuscript designated appendix 2.

5) We have also extended our studies on the genetics of enterotoxin synthesis to enterotoxin SEC_2. We have examined the plasmid DNA profile of 3 SEC_2 isolates and have found that these strains contain only a large plasmid (17.5×10^6 daltons) which is responsible for resistance to cadmium. In relationship to patterns already established we presume this is tentative evidence of a chromosomal locus for SEC_2.

FOREWARD:

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.



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REPORT:

The contract was activated on 1 March 1979 and therefore this report covers the period from the activation date to the present (7 August 1979); a period of 5 months. In spite of this short time we have been able to produce two papers.

1) Shafer, W.M. and J. J. Iandolo. 1979. The genetics of staphylococcal enterotoxin B in methicillin resistant isolates of staphylococcus aureus. Infection and Immunity 25: 902-911.

2) Shafer, W. M. and J. J. Iandolo. 1980. Transduction of enterotoxin B synthesis: Establishment of the toxin gene is a recombination deficient mutant. Infection and Immunity 27: 280-282.

In addition two contributed papers were presented at scientific meetings; one before the American Society for Microbiology and one before the Missouri Valley Branch of the ASM. The paper given in Los Angeles (ASM) was titled:

Shafer, W.M. and J.J. Iandolo. 1979. Demonstration of plasmid and chromosomal genes responsible for staphylococcal enterotoxin B synthesis in methicillin resistant isolates. Bacteriol. Proc. p. 214 (abst.)

1. Statement of the problem:

The enterotoxins produced by the Gram positive pathogenic bacterium, Staphylococcus aureus are a group of extracellular proteins (1, 2) that are responsible for the clinical symptomology of staphylococcal food poisoning. Although many cases of staphylococcal food poisoning probably go unreported, epidemiological data reveals that these enterotoxins account for over 25% (1) of all reported food poisoning incidents. While the immediate manifestations of staphylococcal food poisoning are obvious to the clinician, any additional roles that these proteins play during infection remain unknown. However, enterotoxigenic strains have been isolated from patients with chronic osteomyelitis (3), pseudomembraneous enterocolitis (4) and scalded skin disease (5, 6).

Research efforts to date have been primarily concerned with enterotoxin purification (7, 8, 1, 9, 10, 11. 12. 13), detection (14, 9, 15, 16, 17, 18) and the regulation of toxin synthesis (19, 20, 21, 22, 23, 24). While these studies have contributed a considerable amount of information regarding enterotoxin biochemistry and synthesis, they have not advanced understanding of the molecular genetics of toxin synthesis. Consequently, there exists a dearth of information regarding the genetics of enterotoxin synthesis. Such information could reveal molecular interrelationships of the various enterotoxins, the transmissibility of the enterotoxigenic phenotype and linkage relationships with genes responsible for antibiotic resistance or in the production of proteins involved in pathogenesis.

The present study was undertaken in order to clarify some aspects of the molecular genetics of enterotoxin synthesis. Although six distinct enterotoxins have been purified, enterotoxin B (SEB) was chosen for genetic analysis because previous work by others (25, 26, 27) suggested that the SEB structural gene was extrachromosomal and was linked to genes responsible for antibiotic resistance.

The results obtained during this study reveal that the SEB gene is capable of existing in two distinct configurations. In a majority (71%) of enterotoxigenic isolates, the gene was found to be restricted to a chromosomal locus whereas in other strains, notably hospital isolates resistant to methicillin, the gene gave evidence of being associated with a 1.15×10^6 dalton plasmid deoxyribonucleic acid (DNA) species. Additional studies demonstrated that the toxin phenotype and by implication, the toxin gene, could be transmitted to previously nontoxigenic strains of S. aureus. The establishment of the enterotoxin gene appeared to be contingent upon the concommitant transfer of genes responsible for methicillin and tetracycline resistance. Although genetic studies demonstrated that the enterotoxin B gene is not closely linked to the antibiotic resistance genes, the data obtained suggest that these genes are capable of a transient association.

2. Background

Early evidence suggesting a plasmid locus for the enterotoxin B gene (entB) was provided by Sugiyama et al. (28). They noticed that when enterotoxigenic isolates which produced a single serotype were plated onto agar containing specific antiserum the SEB^+ or SEC^+ colonies produced very

large immune precipitate halos that varied in size. However, SEA^+ colonies gave comparatively smaller halos which did not vary in diameter. The size heterogeneity of the SEB and SEC immune precipitate halos suggested that these phenotypes were variable and hence unstable while the constant size of the SEA halos indicated that this phenotype was stable. These observations were consistent with other reports which have demonstrated that mutants producing variable amounts of SEB are obtainable without mutagenesis (i.e., occur spontaneously) (29). Conversely, Friedman and Howard (30) were able to obtain mutants which produced high amounts of SEA only after repeated mutagenesis.

Based on the apparent phenotypic stability of SEA synthesis and the instability of SEB and SEC synthesis, Bergdoll et al. (2) proposed that SEA is a chromosomal gene product while the other two serotypes were thought to be plasmid gene products. Subsequent work from my laboratory has confirmed a chromosomal locus for entA (31). However, the genetics of SEB synthesis is more complex (25, 32, 33, 27) and will be discussed in more detail. The genetics of SEC_1 and SEC_2 synthesis remain largely unresolved.

Conceptually, entB could exist in three distinct configurations. The gene could be chromosomal, plasmid or as part of a prophage genome. Each of these three possibilities have been investigated.

A bacteriophage involvement in toxin synthesis in other genera is well documented and is perhaps best typified by the diptheria toxin model (34). Specifically, the structural toxin gene is present on a bacteriophage genome. Lysogenization of nontoxigenic strains of Cornybacterium diptheriae confers toxin synthesis and the loss of toxin synthesis is correlated with the loss of immunity to superinfection. Both examples are suggestive evidence of a prophage involvement in toxin synthesis. However, the in vitro synthesis of diptheria toxin using phage DNA directly demonstrated that the toxin is coded by viral DNA sequences (35). Evidence for a phage involvement in the synthesis of the Escherichia coli heat labile (LT) enterotoxin has also been presented (36).

Against this background, the potential for a phage involvement in SEB synthesis should be addressed. Read and Pritchard (37) examined this question and concluded that although enterotoxigenic staphylococci are lysogenic there does not appear to be a phage involvement in toxin synthesis since lysogenization of nontoxigenic recipients does not confer SEB synthesis. Although fairly detailed their work does not absolutely rule out the possibility of a defective phage involvement since their assay system was restricted to inducible prophage. Nevertheless, the overwhelming data concerning the genetics of SEB synthesis indicates that in at least a majority of strains the determinant is bacterial. However, the recent finding of Takeda and Murphy (36) that the LT enterotoxin gene has migrated from a plasmid to a phage genome in at least two enterotoxigenic strains may provide a renewed stimulus for the further examination of a phage involvement in SEB synthesis.

Initial reports regarding the physical disposition of the enterotoxin B gene (entB) were provided by Dornbusch et al. (25, 38). This group analyzed SEB synthesis in the methicillin resistant (Mec^r) isolate, S. aureus DU-4916. Genetic manipulations involving transduction and elimination of Mec^r suggested linkage of entB with the resistance determinant (mec).

Specifically, methicillin sensitive (Mec^S) recipients rendered Mec^r by transduction virtually always produced SEB. Additionally, all Mec^S derivatives, obtained by cultivation in the presence of the curing agent acriflavine, lost the SEB phenotype. Based on these results, Dornbusch et al. (25, 38) proposed that entB and mec are linked on a plasmid. Unfortunately, they did not provide any biophysical data to substantiate the presence of such a plasmid species.

The finding of a potential plasmid involvement in Mec^r provided a stimulus for other studies which were devoted to the elucidation of the genetics of resistance. Cohen and Sweeney (39) reported that unlike the findings of Dornbusch et al. (25, 38) the Mec^r phenotype gave evidence of being coded for by a chromosomal gene since the phenotype could not be eliminated. Stiffler et al. (40) and Kayser et al. (41) examined the extrachromosomal DNA profile of Mec^r isolates and resistant transductants but were unable to isolate a plasmid DNA species equatable with resistance. These groups suggested that unless mec is harbored by a unique plasmid, which is not detectable by standard plasmid identification techniques, the gene should be considered chromosomal.

The most convincing data implicating a chromosomal mec gene was provided by Sjöström et al. (42) and Kuhl et al. (43). Sjöström et al. demonstrated that chromosomal DNA but not plasmid DNA preparations were effective in transforming Mec^S recipients to the resistant phenotype. Additionally, mec transformation occurred in both $recA^+$ and $recA^-$ recipients. Regardless of the recipient, the gene was apparently established in the chromosome since no plasmid DNA equatable with Mec^r could be isolated from the Rec^- Mec^r transformants. Since recombination did not require a functional recA gene product, Sjöström et al. (42) stated that although mec is chromosomal it should be considered a pseudoplasmid, as defined by Wyman et al. (44).

Kuhl et al. (43) confirmed the chromosomal disposition of mec and established that the gene, regardless of the isolate, always maps within linkage group II of the staphylococcal chromosome. By a series of three factor crosses mec was shown to map next to the determinants responsible for novobicin resistance and purine biosynthesis. Because of this linkage arrangement, Kuhl et al. questioned whether the determinant was capable of transposition, as proposed by Sjöström et al. (42). Unfortunately, they did not employ $recA^-$ recipients and as such, the requirement for a functional recA gene product was not ascertained. Since the staphylococcal chromosome remains poorly defined their data does not eliminate the possibility that mec may also exist in other areas of the genome.

None of the previously described studies involved in detailing the genetic locus for Mec^r employed SEB as a secondary marker and the proposed linkage (25, 38) of these two determinants was not resolved. Since the original reports of Dornbusch et al. (25, 38), other reports (27, 31, 33, 27) have appeared dealing with the genetics of SEB synthesis. These studies have been concerned with ascertaining the disposition of the entB gene (i.e., plasmid or chromosomal) and the linkage arrangement of entB and mec. The cumulative data suggests that the genetics of SEB synthesis is quite complicated in that the toxin determinant is capable of exhibiting genetic but not physical linkage with genes responsible for Mec^r and tetracycline resistance (Tc^r) (33, 27) and can exist in either the plasmid and chromosomal state (32, 33, 38).

The work of Shalita et al. (27) represented the first indepth biochemical analysis of the genetics of SEB synthesis. Like Dornbusch et al. (25, 38), this group examined entB in strain DU-4916. Extrachromosomal DNA analysis by neutral sucrose gradient centrifugation of labeled cleared lysates demonstrated that strain DU-4916 harbors three distinct plasmids; 37S, 21S, and 14S. Contour mapping of the 14S plasmid revealed that the molecule had an apparent molecular weight of 0.75×10^6 daltons. Previous investigators (45, 42, 40) did not report the presence of this plasmid in strain DU-4916 but careful inspection of their data indicaces that gradient centrifugation profiles contain this small plasmid, which may have appeared as a "shoulder" of the 21S plasmid.

Marker analysis performed by transduction and elimination demonstrated that the 37S plasmid harbored determinants responsible for penicillin and cadmium resistance while the 21S molecule was responsible for tetracycline resistance (Tc^r). Transduction studies demonstrated that neither entB or mec is linked to either of these plasmids. Ethidium bromide curing experiments, however, demonstrated that the loss of either Mec^r or Tc^r, or both, resulted in the concommitant loss of SEB synthesis. Plasmid DNA analysis of the SEB^- derivatives showed that all of the clones lost the 0.75×10^6 dalton plasmid. Extrachromosomal DNA analysis of various transductants demonstrated SEB^+ clones contained the 0.75×10^6 dalton plasmid species while the SEB^- clones contained only the various antibiotic resistance plasmids. Based on the plasmid DNA analysis of the entB genetic derivatives, Shalita et al. proposed that the 14S plasmid contains a genetic determinant critically involved in SEB synthesis.

Since the SEB^+ phenotype can not be directly selected in genetic manipulations it is possible that the presumptive entB plasmid only harbors a determinant involved in the regulation of toxin synthesis and not necessarily the structural entB gene. To be sure, the inability to directly select for enterotoxigenic clones has proved to be a major hinderance in studies dealing with entB genetics.

Shafer and Iandolo (32) have confirmed the existence of a 14S plasmid in strain DU-4916 and demonstrated that this extrachromosomal species is absent in a nontoxigenic methicillin sensitive derivative (DU-4916S). Velocity centrifugation of labeled cleared lysates in neutral sucrose gradients indicated that the putative entB plasmid has an apparent molecular weight of 1.15×10^6 daltons. A similar molecular weight was obtained when the bulk plasmid DNA obtained from DU-4916 were electrophoresed with staphylococcal plasmids of known molecular weight in agarose gels (33).

Shafer and Iandolo (32) reported that although entB appears to be associated with the 1.15×10^6 dalton plasmid in strain DU-4916 the gene also exists as a chromosomal entity in other isolates. Analysis of the plasmid DNA profile of five methicillin sensitive SEB^+ isolates failed to reveal the presence of the putative entB plasmid. Moreover, genetic examination of the plasmid DNA harbored by these strains failed to reveal association of entB with any of the resident plasmid DNA species. The combined biophysical and genetic results indicated that these isolates contained a chromosomal entB gene.

The reports of Dornbusch et al. (25, 38), Shalita et al. (27) and Shafer and Iandolo (32, 33) have established that entB can exist in either the plasmid or chromosomal state. However, the relationship between mec and entB is less clear. Cotransduction and coelimination results strongly argue that both genes are closely linked. However, the rigorous examination of mec in S. aureus DU-4916 unequivocally demonstrated that the gene is chromosomal (43, 42), while, entB appears to be harbored by the 1.15×10^6 dalton plasmid. Based on the apparent physical disposition of these two genes, the observed linkage appears to be paradoxical.

3. Approach to the problem

In the initial portion of this work, we addressed the question of linkage of methicillin resistance and enterotoxin B production using standard genetic techniques consistent with the state of the art in the staphylococcal genetic system. Transductional and transformational crosses were carried out to sort out the very complex relationships involved. All these were verified by biophysical analysis of the recipient derivatives.

The first question asked was whether the putative enterotoxin B plasmid was common to methicillin resistant strains of S. aureus. To answer this question a survey of the plasmid make-up of 16 mec^r SEB^+ strains was performed. Detailed genetic analysis of four of these strains was carried out by transduction and transformation. Because the data suggested that entB may be a transposon further studies involving a recombination deficient mutant were carried out to test this possibility.

We also wished to know whether one of the sequellae of natural drug transmission among these strains was the co-transfer of toxin determinants. To answer this question we co-cultivated clearly distinguishable genetically marked strains, one of which (the donor) produced SEB and selected for toxin producing variants of the recipient.

Lastly we began a minimal effort to determine the genetic make-up of enterotoxin C producing strains. We approached this in a manner identical to that which we published earlier for SEB (32).

4. Results and discussion

The details of the genetic analysis of entB is contained in Appendices 1 and 2. The reader is directed to these for a more in-depth discussion. However, a summary of these findings is presented here.

Biophysical and genetic analysis (appendix 1) of staphylococcal enterotoxin B (SEB) synthesis in 16 methicillin-resistant (Mec^r) S. aureus isolates demonstrated that the toxin gene (entB) can occupy either a plasmid or a chromosomal locus. Biophysical analysis of the plasmid DNA content of these strains by agarose gel electrophoresis revealed the presence of a 1.15 megadalton (MDAL) plasmid in six isolates (37.5%) that appears to contain the entB gene. Genetic manipulation of SEB synthesis by transduction and elimination demonstrated that this plasmid is critical for enterotoxigenesis. Nevertheless, the majority of the Mec^r SEB^+ isolates (63.5%) analyzed in this investigation were found to lack the 1.15 MDAL plasmid. In at least two of these strains (COL and 57-dk) transduction and elimination showed that entB was chromosomal.

Genetic studies involving strains harboring either a plasmid or a chromosomal entB gene (appendix 1) demonstrated that toxin synthesis is coeliminated with mec. However, analysis of the entB and mec loci by transformation or transduction showed that the genes are not closely linked. On the other hand, transduction of entB, regardless of the donor, was observed when mec and Tc^r (tetracycline) plasmid were jointly cotransduced. This finding suggests that during transduction a transient association among entB, mec and the Tc^r plasmid may exist. Because of the genetic duality (plasmid-chromosomal) demonstrated by entB and since the methicillin gene has already been reported (42) to be part of a transposition-like element due to its ability to be established in recombination deficient hosts ($recA1^-$), we undertook experiments to determine if entB exhibited recA1 independent recombination (Appendix 2). The results of these experiments demonstrated that only entB from strains containing pentB could be established in $recA1^-$ hosts. Indeed the plasmid profile of such recipients clearly showed the presence of pentB. Chromosomal entB strains could not be successfully employed as donors. Therefore, by this criterion it seems that entB does not exhibit properties of a transposon. However, because of the inability to directly select for pentB this experiment may not be entirely adequate to analyze the question of entB transposability. On the other hand, the high cotransfer of entB, mec and tc (85% of mec^r Tc^r clones are SEB^+) argue strongly for the validity of the approach.

In as much as the entB gene could not be shown to undergo recA1 independent recombination we attempted to map the entB gene on the S. aureus chromosome. We had shown (Appendix 1) that entB did not map near mec in linkage group II but had empirically noted that most SEB^+ transformants were also transformed for pigment production (pig^+). We therefore examined the pig region of linkage group III. DNA from strain COL was used to transform ISP484 (33). Recombinants were selected that were prototrophic for isoleucine, leucine and valine (ilv) and scored for pigmentation. Ilv was transformed at a frequency of 3.8×10^{-6} (38 total clones). Cotransformation of pig was 8% (3 clones) and all of these were SEB^+. The data thusfar are encouraging and hint that entB may map between ilv and pig. However expression of toxin is strongly subdued (that is; 25X concentration of the medium must be made before SEB can be detected) and transformant clones produce only about 1 µg of SEB/ml. We are continuing mapping studies and studies designed to clarify the genetic associations alluded to in appendix 1.

In an associated study we wished to know whether one of the sequellae of natural drug transmission among these strains was the co-transfer of toxin determinants. To answer this question we co-cultivated clearly distinguishable genetically marked strains, one of which (the donor) produced enterotoxin B. The relevant phenotype of the donor was mec^r tet^r nov^s SEB^+ (ϕ11), the phenotype of the recipient was mec^s tet^s nov^r SEB^-. Recombinants were scored for the mec^r tet^r nov^r SEB^+ phenotype. Although transduction frequencies as high as 2×10^4 recombinants per plaque forming unit were observed for single markers, the frequency of the triple transductants was considerably lower but nonetheless did occur. Since lysogenized staphylococci exhibit relatively high amounts of prophage leakiness, this result is not unexpected. However the high frequencies observed suggest that a higher proportion of leaky phage are transducing particles than are phage from conventional lysates. Furthermore, this result provides evidence of genetic promiscuity among naturally associated strains and may provide the mechanism for the contribution to or exacerbation of other staphylococcal disease by enterotoxin.

We have begun studies to investigate the genetic disposition of enterotoxin C in S. aureus. Through the generous gift of SEC_2 from Dr. R. Bennett (FDA Washington, D.C.) we have prepared antibody for analysis in a manner similar to that reported by us for SEB (32). SEC_1 and SEC_2 can be analyzed with this serum by Laurell gel electrophoresis with about the same sensitivity as SEB. SEC_1 and SEC_2 can be distinguished in our systems because SEC_1 cross reacts with antiserum to SEB whereas SEC_2 does not.

Three strains were initially chosen for analysis (strains 735, 740 and 834. These strains were also obtained from Dr. R. Bennett, FDA, Washington). Strains 735 and 834 produced both SEC_1 and SEC_2 while strain 740 produced SEC_2. All three produced enterotoxin C at about 50 µg/ml. Biophysical analysis of the plasmid DNA of these three strains by CsCl·ethidium bromide centrifugation followed by agarose gel electrophoresis demonstrated that all three possess a single plasmid species of 17.5×10^6 daltons. Analysis of this plasmid in all three strains has shown it to confer cadmium and penicillin resistance. In relationship to patterns already established we presume this is tentative evidence of a chromosomal locus for SEC_2. Work is continuing on a more critical analysis of SEC production.

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